

## THE DETECTION OF *Plasmodiophora brassicae* USING LOOP-MEDIATED ISOTHERMAL DNA AMPLIFICATION

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### Abstract

*Plasmodiophora brassicae*, the cause of clubroot, is a very serious problem preventing from successful and profitable cultivation of oilseed rape in Poland. The pathogen was found in all main growing areas of oilseed rape; it also causes considerable problems in growing of vegetable brassicas. The aim of this work was to elaborate fast, cheap and reliable screening method to detect *P. brassicae*. To achieve this aim the Loop-mediated isothermal DNA amplification (LAMP) technique has been elaborated. The set of three primer pairs was designed using LAMP software. The detection was performed with the GspSSD polymerase, isolated from bacteria *Geobacillus* sp., with strand displacement activity. DNA extraction from clubbed roots obtained from farmers' fields of oilseed rape infected by *P. brassicae* was done using a modified CTAB method. The reaction was performed for 60 min at 62°C. The visual detection was done using CFX96 Real Time PCR Detection System (BioRad) or Gerie II Amplicator (Optigen). The detection with LAMP proved its usefulness; it was easy, fast and accurate and independent of plant age. The detection limit was 5 spores per 1 µl of the spore suspension, so LAMP was less sensitive than quantitative PCR tests reported in the literature. However, the method is cheap and simple, so it is a good alternative, when it comes to practical use and the assessment of numerous samples.

**Key words:** clubroot; isothermal DNA amplification; *Plasmodiophora brassicae*; oilseed rape; GspSSD polymerase; strand displacement activity; loop-mediated isothermal DNA amplification, LAMP.

### INTRODUCTION

*Plasmodiophora brassicae* Woronin is an obligate biotrophic pathogen of crops belonging to Brassicaceae family [1,2]. According to the recent phylogenomic analyses based on ESTs it belongs to the group of Phytophyta, which is a part of the supergroup of

Rhizaria. This taxonomic group is regarded as one of the most poorly understood supergroups of eukaryotes [3]. However, newly available transcriptome studies allowing to perform phylogenomic analyses of very small amounts of material provided deeper insights in early evolution of eukaryotes and generated large amounts of data [4]. The studies include parasitic protists, such as *P. brassicae*. Other important plant pathogens, such as *Spongospora subterranea* – the causal agent of powdery scab on potato, also belong to Rhizaria and Phytophyta. Both pathogens are found worldwide, where the host plants are consistently grown, and their effect on yield is high. They either directly reduce the amount of collected seeds of cruciferous plants, as is the case of *P. brassicae*, or the effect on yield is indirect, through the unmarketable, scabbed appearance of potato tubers, as for *S. subterranea*.

*Plasmodiophora brassicae* is responsible for clubroot, the disease known on cruciferous crops in Europe since at least the XIII century [1]. Subsequently, the pathogen probably spread worldwide on and in fodder taken by colonists as livestock feed [2]. In Poland for many years it was posing a serious threat to vegetable brassicas [5]. However, in recent time clubroot is a very serious problem preventing from successful and profitable cultivation of oilseed rape [6].

Spring canola and winter oilseed rape are essential sources of vegetable oil in human and livestock foodstuffs [2], as well as industrial products such as biodiesel and lubricants [7,8]. The crop is bringing one of the highest profits to farmers in all important growing areas of oilseed rape located in Europe, Canada, Australia, China and India, so a disease that decreases its yield is highly undesirable. Also in Poland, the problem is serious as the disease is present in all agronomic regions with a high share of oilseed rape in a sowing

structure [6]. The pathogen is very persistent in soil [9], but until recently the spread of virulent *P. brassicae* pathotypes was regarded as relatively slow [10].

Agricultural practice and numerous research studies require the knowledge about the presence and concentration of the pathogen in plants, soil and water. Recent studies of primary and secondary infection processes by *P. brassicae* on canola showed that early stages of infection of canola roots required  $1 \times 10^5$  resting spores  $\text{mL}^{-1}$  for primary (root hair) infection at 12 h after inoculation, whereas secondary (cortical) infection began to be observed 3 days after inoculation. When inoculated onto plants at a concentration of  $1 \times 10^4$   $\text{mL}^{-1}$ , secondary zoospores produced primary infections similar to those obtained with resting spores at a concentration of  $1 \times 10^5$   $\text{mL}^{-1}$  [11]. In general, the level of  $10^3$  spores per 1 g of soil is regarded as the concentration of the spores of *P. brassicae* required to produce symptoms on plants and the level of  $3 \times 10^3$  *P. brassicae* resting spores  $\text{g}^{-1}$  soil, is suggested for growing resistant cultivars [12]. Cultivation of resistant varieties has been by now regarded as the most effective method of controlling clubroot in many cropping areas [10]. In agricultural practice it is also important to make sure that clubs on roots are resulting from the infection by *P. brassicae* and not from other reasons, such as the damage caused by gall-forming root weevils *Ceutorhynchus pleurostigma* Marsham [= *C. assimilis* Paykull] or *C. cardariae* Korotyaev.

To achieve this aim different molecular tests has been proposed by some researchers. It was already nearly 20 years ago when the single copy DNA sequence unique to *P. brassicae* have cloned [13] and then used to develop a nested PCR-based technique allowing to detect *P. brassicae* resting spores in soil [14]. At the same time a similar technique, with the use of specific PCR primers was also proposed for the detection of the pathogen not only from soil, but also from water samples [15]. In 2001 the technique of nested PCR was successfully used in Poland to prove the usefulness of the treatment of field soil and peat moss substrate with IBE 3878 fungicide or hot steam. Both treatments lowered the concentration of resting spores below  $10^3$  per 1  $\text{g}^{-1}$  of soil, what was the detection limit of this method [16].

In 2007, Cao et al. [17] developed a simple, one-step PCR-based protocol allowing to detect the pathogen in plant and soil samples, whereas in recent years two quantitative PCR protocols were proposed [12,18]. The method by Wallenhammar et al. [12] used species-specific primers and a TaqMan fluorogenic probe designed to amplify a small region of *P. brassicae* ribosomal DNA. The detection limit in soil samples corresponded to 500 resting spores  $\text{g}^{-1}$

soil. The detection limit of the method described by Li et al. [18] was lower as it corresponded to  $10^3$  resting spores  $\text{g}^{-1}$  soil, but the method was cheaper, as it was based on a SYBR Green chemistry and species-specific primers of *P. brassicae*, amplifying the conservative region of rDNA-ITS. Both tests, although very specific and sensitive require many steps of DNA purification and expensive reagents as well as high laboratory skills. The aim of this work was to elaborate fast, cheap and reliable screening method to detect *P. brassicae*, for its further use in different substrates, such as plants, soil and water.

## MATERIALS AND METHODS

### Plant sampling

Plant samples were collected from glasshouse experiments carried out to evaluate the pathotypes of *Plasmodiophora brassicae*, based on the system designed by Somé et al. [19]. Differential reaction on *Brassica napus* ECD06, ECD10 and cv. Brutor assigned tested single-club isolates to five groups P1 to P5. A clubbed root from each pathotype was then homogenised using high-performance Ultra Turax T25 Digital homogeniser with S25N-10G and S25-18G dispersing elements. The spores were adjusted to  $5 \times 10^6$  per 1 mL of the spore suspension and then mixed in equal proportions. They were then used to assess the resolution of the method. The test was also performed using five field isolates of *P. brassicae*, collected in different regions of Poland: 1) Siemyśl (N 54°01'38.9' E 15°31'57.3'), West Pomerania; 2) Bielnik (N 54°11'08.0' E 19°21'51.8'), Varmia; 3) Kobierno (N 51°43'19.1' E 17°29'07.4'), Great Poland; 4) Wrzesiny (N 51°42'11.7' E 15°26'02.6'), Lubusz region; 5) Gorzanowice (N 50°56'38.9' E 16°04'57.6'), Lower Silesia. The location of these sites is shown in Fig. 1.

### Extraction of DNA

To extract DNA of *P. brassicae* the solution of resting spores was placed in Eppendorf tubes with 20 mg of SiC and 650  $\mu\text{L}$  CTAB (hexadecyltrimethylammonium bromide) extraction buffer. The CTAB buffer contained 100 mM TRIS-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl. The mixture was heated at 65°C for 20 min. and then it was shaken with  $\text{CHCl}_3$ . The extract was acidified with  $\text{CH}_3\text{COOK}$  (3M, pH 5.4), it was kept on ice and then vortexed. The aqueous phase was collected and DNA was extracted using pure ice cold ethanol (99.8%). The sediment was dissolved in TE (10 mM Tris-HCl, 0.1 mM EDTA) buffer and this solution was used for LAMP reactions. To test the resolution of the method the 10 $\times$ , 100 $\times$  and 1.000 $\times$  dilutions of template DNA were made.



Fig. 1. The origin of the field samples of *Plasmodiophora brassicae* (clubbed roots) from different regions of Poland used in this study.

#### Pathogen detection by LAMP method

The LAMP assay was performed using 12.5- $\mu$ L a reaction mixtures containing 1  $\mu$ L of template DNA or its 10 $\times$ , 100 $\times$  and 1.000 $\times$  dilutions. The reaction mix contained 1.6  $\mu$ M each of inner primers FIP and BIP, 0.2  $\mu$ M each of outer primers F3 and B3, and 0.8  $\mu$ M of loop primers LF and LB, Isothermal Mastermix – fluorescent dye 1x (Optigene, UK). The reaction mixture was incubated at 62°C for 60 min and then at 85°C for 2 min to complete the reaction. The visual detection

was done using CFX96 Real Time PCR Detection System (BioRad) or Gerie II Amplificator (Optigene).

#### RESULTS

The detection of the DNA extracted from the mixture of resting spores of P1–P5 pathotypes of *P. brassicae* was successful using the described protocol. The LAMP product was generated in case of all used dilutions of the DNA template (Fig. 2).

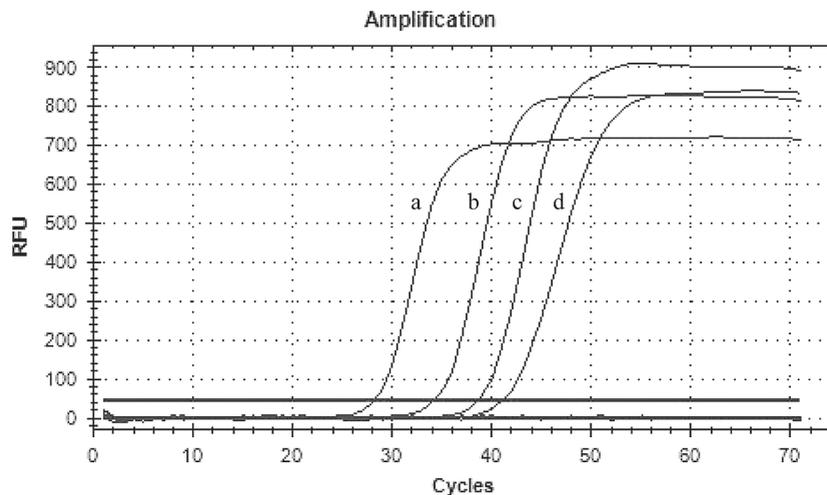


Fig. 2. Resolution of Loop-mediated isothermal DNA amplification (LAMP) of *Plasmodiophora brassicae*, using 1  $\mu$ L of: a) template DNA, b) 10 $\times$  dilution of template DNA, c) 100 $\times$  dilution d) 1000 $\times$  dilution of template DNA.

The melting curve was of appropriate shape, without any additional peaks due to unwanted LAMP products or primer dimers (Fig. 3).

The LAMP method was also successful in the detection of the pathogen from randomly collected plant root samples originating directly from fields infected by *P. brassicae*, collected in different regions of Poland.

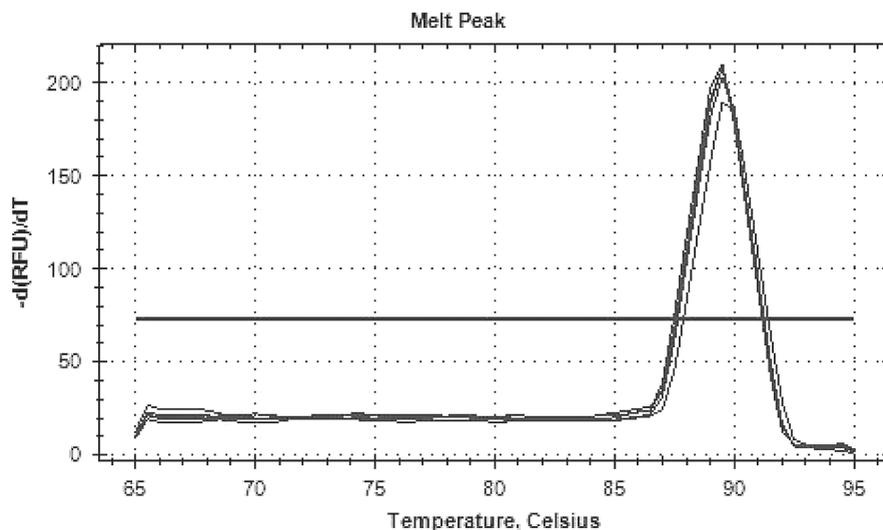


Fig. 3. Melting curve of the Loop-mediated isothermal DNA amplification (LAMP) of *Plasmodiophora brassicae* product (ITS-based).

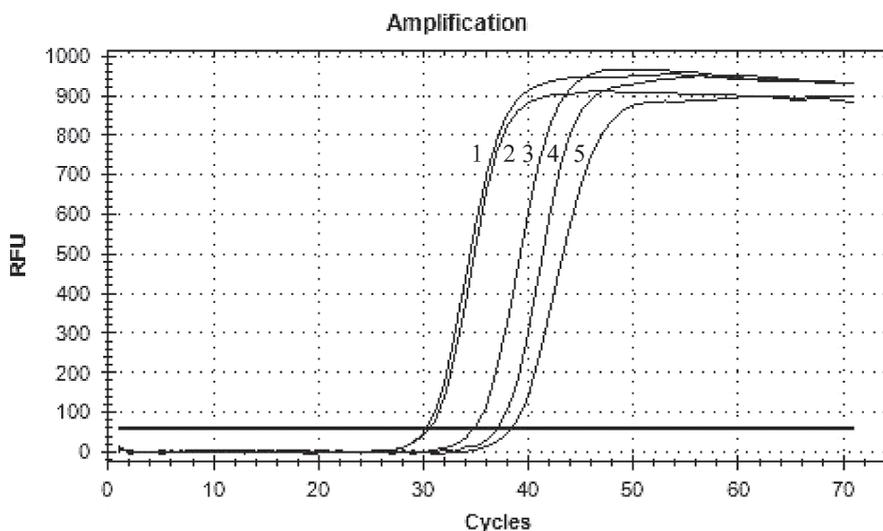


Fig. 4. Detection of *Plasmodiophora brassicae* from various samples using LAMP method: 1) Bielnik, Varmia; 2) Gorzanowice, Lower Silesia; 3) Siemyśl, West Pomerania; 4) Wrzesiny, Lubusz region; 5) Kobierno, Great Poland.

## DISCUSSION

Serious problems with high incidence and very big severity of clubroot on Brassicaceae crops, especially on cultivated brassicas, require quick, sensitive but robust methods of pathogen detection. In case of uncultivable microorganisms, such as *P. brassicae*, belonging to Rhizaria, the visual recognition in clubbed roots is a too late step to undertake the appropriate measures to control the disease. The technique of Loop-mediated isothermal DNA amplification has numerous

advantages that seems beneficial to monitor the disease development in its early steps: it allows to perform the reaction in a quick manner, using simple tools and relatively cheap reagents [20,21], and it is very sensitive due to the use of loop primers [22].

Pathogen populations, even the ones belonging to the same species usually greatly differ in many characters [23–25]; so is the case of *P. brassicae*, where a large variation of pathotypes and physiological specialization has been reported [26–28]. LAMP method proved its usefulness for the detection of resting spores

of *P. brassicae* of different origin. The experiments were successful both using infected plant material obtained from pure single-club isolates propagated in glasshouse conditions and infected plants obtained from farmers' fields located in different regions of Poland. The sensitivity of the method is *ca.* 5.000 spores per 1 mL, so it is lower as compared to qPCR methods described in the literature [12,18], but it is sufficient for practical use. The lower detection limit is compensated with simplicity and low costs of testing, which are particularly preferred for everyday use and studies of numerous samples. It seems that the next necessary step to go is to develop pathotype-specific LAMP markers. A SCAR marker OPL14<sub>1200</sub> was already reported to detect P1 pathotype of *P. brassicae* [29], but the method was based on end-point PCR, so its sensitivity is most probably not sufficient enough for monitoring of minute amounts of the pathogen.

So far, species-specific detection using LAMP was proposed for some closely related species, such as *Leptosphaeria maculans* and *L. biglobosa* [30] or *Nosema apis* and *N. ceranae* [31], as well as methicillin-resistant *Staphylococcus aureus* (MRSA) in blood cultures [32] and virus subtype H5N1 of avian influenza [33]. The method proved its tolerance to culture media and biological substances, that can inhibit other molecular methods [34], so it seems that more pathogen detection tests will be soon developed, as it was forecasted by Tomlinson and Boonham [35]. These methods may be implemented and widely used not only in research studies but also by extension and sanitary services.

## CONCLUSION

A loop-mediated isothermal DNA amplification method was developed for a rapid and sensitive detection of *Plasmodiophora brassicae*, a damaging pathogen causing clubroot of oilseed rape and vegetable brassicas. The method is cheap and simple, so it is a good alternative to quantitative PCR, when it comes to practical use for the assessment of numerous samples.

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## Authors' contributions

Concept of the study: MJ; glasshouse experiment: MJ; design of primers: AB; DNA extraction and pathotype sequencing: WI, LAMP optimization and performance: JK; analysis of research results: MJ, JK; writing of the manuscript: MJ, JK.

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**Wykrywanie *Plasmodiophora brassicae*  
przy zastosowaniu amplifikacji DNA  
w warunkach izotermicznych z wykorzystaniem  
starterów zapętlających (LAMP)**

**Streszczenie**

*Plasmodiophora brassicae* powoduje kiłkę kapusty, chorobę obniżającą plonowanie i redukującą rentowność uprawy rzepaku. Patogen występuje we wszystkich głównych regionach jego uprawy, a także stanowi poważny problem dla producentów warzyw kapustowatych. Celem badań było opracowanie szybkiego, taniego i niezawodnego sposobu wykrywania *P. brassicae*. W tym celu opracowano metodę amplifikacji DNA w warunkach izotermicznych z wykorzystaniem starterów zapętlających (LAMP). Do detekcji wykorzystano

polimerazę GspSSD, wyodrębnioną z bakterii *Geobacillus* sp., umożliwiającą zastępowanie nici DNA bez potrzeby zmiany profilu temperatury. Ekstrakcję DNA z wyrosli na korzeniach wykonano z zastosowaniem zmodyfikowanej metody CTAB. Reakcję prowadzono przez 60 minut w temperaturze 62°C. Wyniki zbierano przy zastosowaniu systemu CFX96 Real Time PCR Detection System (BioRad) lub Gerie II Amplificator (Optigene). Opracowana metoda była łatwa do wykonania, szybka, dokładna i niezależna od wieku badanej rośliny. LAMP był testem mniej czułym aniżeli opisane w literaturze metody ilościowego PCR; umożliwił wykrywanie 5000 zarodników w 1 mL zawiesiny. Jest to jednak metoda znacznie łatwiejsza do wykonania i zdecydowanie tańsza, co czyni ją przydatną do detekcji *P. brassicae* w przypadku konieczności oceny jego obecności w licznych próbach polowych.

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